PECTINOPHORA GOSSYPIELLA (PINK BOLLWORM) *BACILLUS THURINGIENSIS* TOXIN RECEPTOR BT-R_2

CROSS-REFERENCES TO RELATED APPLICATIONS

This Application for Patent claims the benefit of priority from, and hereby incorporates by reference the entire disclosure of, co-pending U.S. Provisional Application for Patent Serial No. 60/161,564 filed October 26, 1999.

TECHNICAL FIELD OF THE INVENTION

This invention generally relates to receptors for *Bacillus thuringiensis* (BT) toxin and thus to pesticides able to bind the receptor, and to ameliorating pesticide resistance. In particular, the invention relates to

recombinant DNA and expression systems for a novel receptor and receptor elements from *Pectinophora gossypiella*, the pink bollworm.

BACKGROUND OF THE INVENTION

Without limiting the scope of the invention, its background is described in connection with uses of *Bacillus thuringiensis* toxins as cotton insect biocidal agents, as an example. Cotton insect pests reduced yields by almost 10% across the US in 1998. Insect damage reduced the overall cotton yield by more than 1.7 million bales and produced a financial loss of about $1.224 billion. One group in particular, the bollworm/budworm complex was the most damaging causing a 2.7% loss. The pink bollworm, *Pectinophora gossypiella* Saunders ("*PBW*"), is a lepidopteran insect that causes severe damage to cotton and is the most destructive pest of cotton worldwide.

Bacillus thuringiensis is a gram positive, sporeforming bacterium that forms a parasporal crystal which contains insecticidal toxins (Bulla et al., *Crit. Rev. Microbiol.* (1980) **8**: 147-204; Höfte and Whiteley, *Microbiol. Rev.* (1989) **53**: 242. The effect of the toxin is mediated through binding

to specific receptors on the apical brush border of the midgut microvillae (BBMV) of susceptible insects.

Biological control of cotton pests using *B. thuringiensis* formulations and transgenic plants has been in use for a number of years and is growing rapidly. Recently, transgenic cotton plants carrying the toxin genes of BT have been developed and sold commercially. Such transgenic plants have a high degree of resistance to the pink bollworm (Schnepf et al., *Microbiol. Mol. Biol. Rev.* (1998) **62**: 775). However, the introduction of any new insecticide into a pest management program immediately initiates a selection process for individuals that are resistant to the pesticide. As the use of transgenic crops expressing BT toxin increases, insect resistance is expected to become more widespread. Increased tolerance for BT toxins in several species of insects has been reported by several investigators while laboratory selection experiments have shown that the use of BT toxin formulations and transgenic plants can provoke the development of resistance in the pink bollworm (Bartlett, et al., *Beltwide Cotton Conference* (1995) **2**: 766).

Concerns that BT toxin formulations or transgenic plants expressing the toxin genes may evoke emergence of either resistant or tolerant strains of insects has made the search

for a better understanding of the interaction between the BT toxin proteins and their respective insect receptors a matter of considerable economic importance.

In U.S. Patent No. 5,693,491, the present inventors disclosed the purification and cDNA cloning of a *B. thuringiensis* toxin receptor BT-R_1 from larvae of the tobacco hornworm *Manduca sexta* (*M. Sexta*). Recently, two BT toxin receptors have been identified, purified and cloned from the silkworm, *Bombyx mori* (Nagamatsu et al., *Biosci. Biotechnol. Biochem.* (1998) **62**: 727).

Heretofore in this field, there has been no structural information concerning the structure and function of BT toxin receptor of the major cotton insect pest, *P. gossypiella*. Furthermore, to the inventors' knowledge, the minimum binding fragment encoding a consensus binding domain for BT toxin on the BT receptor has not yet been identified. Isolation of the minimum binding fragment could permit cloning and structural characterization of important yet uncharacterized BT toxin receptors from other insects of worldwide economic importance such as *P. gossypiella*.

SUMMARY OF THE INVENTION

The present invention provides information and materials for isolation and expression of novel BT crystal toxin receptors, herein referred to as Cry toxin receptors. Generally, the invention provides structural and functional characterization of a novel lepidopteran BT toxin receptor, herein referred to as BT-R_2.

A cDNA that encodes an alternative glycoprotein receptor from the pink bollworm that binds specifically to a *B. thuringiensis* toxin has been cloned, sequenced and characterized. The BT-R_2 cDNA permits the analysis of receptors in pink bollworm and other insects and organisms that affect crop growth and development, as well as the design of assays for the cytotoxicity and binding affinity of potential pesticides. The clone and other methods described herein, permit the manipulation of natural and/or introduced homologous receptors and, thus, to specifically destroy organisms, tissues and/or cells of the target host, including insects resistant to toxins of *B. thuringiensis*.

The invention further provides purified and cloned cDNA encoding a 200 kD receptor for the Cry1A toxins of the pink bollworm, *P. gossypiella*. An advantage of this invention is the identification of the minimum binding fragment encoding

the toxin binding domain on the BT toxin receptor. Another advantage of this invention is the provision of methodologies for cloning and structural characterization of presently unknown BT receptors. Furthermore, this invention provides methods and materials for identification and design of effective toxin binding receptors for use in combating emergence of toxin resistance. Also, this invention may be used to generate transgenic organisms expressing toxin receptors.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete understanding of the method and apparatus of the present invention may be obtained by reference to the following Detailed Description when taken in conjunction with the accompanying Drawings wherein:

FIGURES 1A-B show the nucleotide sequence cDNA encoding the BT-R_2 protein from *P.gossypiella* (SEQ ID NO:1);

FIGURES 2A and 2B show the amino acid sequence of BT-R2 protein from *P. gossypiella* (SEQ ID NO: 2). Arrows indicate the start site of the putative cadherin domains **CR1 - CR12**, **SIG** = signal sequence (double underline); **MPD** = membrane proximal domain; **CYT** = cytoplasmic region. The transmembrane region is underlined and bold. The leucine zipper motif **LZ**

is underlined. Ñ residues denote putative N-glycosylation sites. The minimum binding fragment **MBF** (aa 1269-1367) is also double underlined;

Figure 3A is a graph showing the binding results of Cry1A toxins on *P.gossypiella* larvae brush border membrane vesicles prepared from midgut epithelial cells;

Figure 3B is a graph showing the toxicity results of Cry1A toxins on P. *gossypiella* larvae and BBMV;

Figure 4 is a map of the structure of the pink bollworm (PBW) BT-R_2 cDNAs, including truncations PBW-1210-1439, PBW-1269-1439, PBW-1367-1496, and PBW-1210-1367 (the minimum binding fragment). The binding of proteins expressed from each clone to Cry1A toxin was identified by (+) for binding and (-) for non-binding; and

FIGURES 5A-C illustrate an alignment of the silk worm (top), the tobacco hornworm (middle), and the pink bollworm (bottom) Cry toxin receptors. Perfectly conserved residues are boxed.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EXEMPLARY EMBODIMENTS

The present invention will now be described more fully hereinafter with reference to the accompanying drawings, in

which preferred embodiments of the invention are shown. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

ABBREVIATIONS AND DEFINITIONS

The following abbreviations are used throughout this application: bp - base pairs; BT - *Bacillus thuringiensis* or *B. thuringiensis*; BT-R_x - BT toxin receptor of type x; BBMV - brush border of the membrane vesicles; cDNA - complementary DNA; Cry toxin - parasporal crystalline toxin of *BT;* IEF - immunoelectrophoresis; kb - kilobase or kilo base pairs; kD - kilodaltons; K_d - dissociation constant; LC_{50} - lethal concentration resulting in a 50% mortality; PBW - pink bollworm, *Pectinophora gossypiella* or *P. gossypiella*; PCR - polymerase chain reaction; RACE - Rapid Amplification of cDNA Ends; RT - reverse transcriptase; SW - silkworm (*Bombyx mori* or *B. mori*); THW - tobacco hornworm (*Manduca sexta* or *M. sexta*); and UTR - untranslated region.

The term "x% homology" refers to the extent to which two nucleic acid or protein sequences are identical as determined

by BLAST homology alignment as described by T.A. Tatusova & T.L. Madden (1999), "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS MICROBIOL LETT. 174:247-250 and using the following parameters: Program (blastn) or (blastp) as appropriate; matrix (OBLOSUM62), reward for match (1); penalty for mismatch (-2); open gap (5) and extension gap (2) penalties; gap x- drop off (50); Expect (10); word size (11); filter (off). An example of a web based two sequence alignment program using these parameters is found at http://www. .ncbi.nlm.nih.gov/gorf/bl2.html.

The invention thus includes nucleic acid or protein sequences that are highly similar to the sequences of the present invention, and include sequences of 80, 85, 90, 95 and 98% similarity to the sequences described herein.

The invention also includes nucleic acid sequences that can be isolated from genomic or cDNA libraries or prepared synthetically, and that hybridize under high stringency to the entire length of a 400 nucleotide probe derived from the nucleic acid sequences described herein under. High stringency is defined as including a final wash of 0.2X SSC at a temperature of 60°C. Under the calculation:

Eff Tm = 81.5 + 16.6(log M [Na+]) + 0.41(%G+C) - 0.72(% formamide)

the percentage allowable mismatch of a gene with 50% GC under these conditions is estimated to be about 12%.

The nucleic acid and protein sequences described herein are listed for convenience as follows:

SEQ ID Nos.:	DNA and Protein Sequences
SEQ ID NO: 1	BT-R_2 cDNA sequence from *P. gossypiella* (Figure 1)
SEQ ID NO: 2	BT-R_2 protein sequence for *P. gossypiella* (Figure 2)

SEQ ID Nos.:	Primer Sequences	Primer Name
SEQ ID NO: 3	5' CAN ATH CGN GCN CAN GAY GGN GG 3'	BTR 1209U
SEQ ID NO: 4	5' TTG TAC ACS GCW GGS ATW TCC AC 3'	BTR 1355U
SEQ ID NO: 5	5' NAC YTG RTC RAT RTT RCA NGT CAT 3'	BTR 1486D
SEQ ID NO: 6	5' NCC DAT NAG RTC NGA RTC RTT NGA 3'	BTR 1657D
SEQ ID NO: 7	5' TAG GTT GTA TCC TCA GTA TGA GGA 3'	PBW-BTR GSP-1
SEQ ID NO: 8	'5' CCA GAG TGG AGT CCA CCG CCA TA 3'	PBW-BTR GSP-2

SEQ ID NO: 9	5' CTG AGT AAG TGT TAT CTT GAA AG 3'	PBW-BTR GSP-3
SEQ ID NO: 10	5' CAN ATH CGN GCN CAN GAY GGN GG 3'	BTR 1209U
SEQ ID NO: 11	5' GAT AGC GGC CCC AGG AAC CAA CAA ACA GG 3'	PBW-BTR GSP-4
SEQ ID NO: 12	5' AGT GCG AGT GCT TTG AAT CTG TGA 3'	PBW-B'IR P2U
SEQ ID NO: 13	5' GTC TCT TCT CAC CGT CAC TGT CAC T 3'	PBW-BTR P5U
SEQ ID NO: 14	5' GCA TGC TGG CAG TAG GTT GTA TC 3'	PBW-BTR P6D
SEQ ID NO: 15	5' GGC CAC GCG TCG ACT AGT AC 3'	(AUAP)
SEQ ID NO: 16	5' GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T 3'	(AP)

N = A, C, T, or G; H = A, T, or C; B = T, C, or G; D = A, T, or G; V = A, C, or G; R = A or G; Y = C or T; M = A or C; K = T or G; S = C or G; W = A or T

More particularly, the studies described herein were targeted toward the identification, cloning and characterization of novel Cry toxin receptors. One embodiment was directed to characterization and isolation of the heretofore unidentified Cry toxin receptor of the pink bollworm, *P. gossypiella*, hereinafter referred to as "PBW".

In order to identify and isolate the Cry toxin receptor of the PBW, toxicity was determined for five different Cry proteins (Cry1Aa, Cry1Ab, Cry1Ac, Cry3A and Cry11A) against neonate PBW larvae. It was determined that the lepidopteran-specific toxins (Cry1Aa, Cry1Ab and Cry1Ac) showed high toxicity toward PBW larvae with a LC_{50} ranging from 25-45 ng/cm^3 of insect diet, while the coleopteran specific (Cry3A) or the dipteran specific (Cry11A) toxins did not exhibit any detectable toxicity up to 2000 ng/cm^3 (Figure 3).

The binding of the three lepidopteran-specific Cry1A toxins (Cry1Aa, Cry1Ab and Cry1Ac) to the BBMV of *P. gossypiella* was characterized in detail. Ligand blot experiments showed that proteins of 120 kD bind only the Cry1Ac toxin whereas a 200 kD protein binds to Cry1Aa, Cry1Ab and Cry1Ac toxins. It is now known that the 120 kD protein is a heat shock protein, although its relation to the Cry toxin effect is not understood.

In the case of the 175 kD cadherin-like Cry1Aa binding protein from *Bombyx mori*, ^{125}I -labeled Cry1Aa binding was eliminated by the presence of unlabeled Cry1Aa, but additional band(s) of approximately 110 kD, identified by ^{125}I-Cry1Aa ligand blots, failed to demonstrate a detectable degree of competition. Thus, it was determined that *P.*

gossypiella, like *M. sexta* and *B. mori*, contains *both* high-affinity and low-affinity binding proteins for at least one Cry1A toxin and that the 200 kDa protein from PBW is a common binding protein for the lepidopteran-specific Cry1A toxins.

The detailed mechanism of the Cry1A toxin interaction with the midgut BBMV of the pink bollworm was determined. The equilibrium dissociation constants (K_d) calculated from the homologous competition assays (Figs. 3A and 3B) are 16.5, 12.4 and 12.8 nM and the concentrations of binding sites are 3.7, 3.6 and 8.6 pmol/mg, for Cry1Aa, Cry1Ab and Cry1Ac, respectively. The Hill Coefficients for the three Cry1A toxins are between 0.6 and 0.8 for BBMV binding proteins (Figure 3A), indicating that there is negative cooperativity in the binding of these toxins to the binding site(s) in the BBMV. Binding of the Cry1A toxins to BBMV proteins was specific and saturable. The toxin amount required for saturation of 460 μg of BBMV proteins was in the following order: Cry1Ac>Cry1Aa>Cry1Ab.

Immunoprecipitation of BBMV proteins with anti-Cry1Ab antiserum and subsequent ligand blotting with ^{125}I-Cry1Ab toxin also showed binding of the toxin to an approximately 200 kD protein. The 200 kD protein is a single protein as shown by 2D-gel analysis (data not shown). A comparison

between the 210 kD binding protein from *M. sexta* with a pI ~ 4.3 and the 200 kD binding protein from P. *gossypiella* (pI - 4.1) revealed that both proteins have almost the same pI. It was determined that the 200 kD PBW protein had some cross-reactivity with polyclonal antisera against the *M. sexta* BT-R_1 210 kD protein.

In order to clone the PBW BT-R_2 gene, fully degenerate primers were designed based on the conserved amino acid sequences between that of the two receptors, tobacco hornworm ("THW") BT-R_1 and silkworm ("SW") BT-R175. The primer locations were designed to include or exclude a sequence thought by the present inventors to encode a region in the extracellular domain critical to toxin binding, herein after "READ" signature sequence. Hereinafter this binding fragment of the DNA sequence will be referred to as the "signature" region.

Three clones were obtained, PBW-421 (aa 1367-1496), PBW-866 (aa 1210-1496) and PBW-1373 (aa 1210-1675), which have about 50 % nucleotide and about 60% amino acid sequence similarity to both THW BT-R_1 and SW BT-R175. The 421 bp and 866 bp clones encode proteins of about 21 and 32 kD, respectively. Although both expressed proteins cross-reacted with THW BT-R_1 polyclonal antisera, the 32 kD protein, but

not the 21 kD protein, was shown to bind Cry1Ab toxin specifically with high affinity. The estimated K_d value is about 17 nM, which is similar to the K_d value obtained for BBMV. Similarly, an internal fragment from the PBW-866 clone did not bind toxin, but did cross-react with BT-R_1 antibodies. This data demonstrates that recognition by anti-BT-R_1 antibodies is insufficient to define a functional toxin receptor.

In order to obtain a cDNA sequence encoding the full-length receptor, the 5' and 3' ends of the PBW BT-R_2 receptor were first obtained using 5' and 3' RACE reactions followed by cloning of the full-length receptor cDNA using gene specific primers from the 5' and 3' UTR. The full-length cDNA clone (SEQ ID NO: 1) has an open reading frame of 1729 amino acids (SEQ ID NO:2), with a deduced molecular weight of 194 kD and a calculated pI value of 4.1, which is similar to the value determined by 2-D gel analysis.

The protein consists of three domains: extracellular, transmembrane and cytoplasmic. The protein sequence contains two hydrophobic regions, one at the amino terminus, characteristic of a signal peptide and one near the COOH-terminus (amino acids 1575-1600) that probably forms a transmembrane domain. The extracellular domain contains 12

cadherin-like motifs, in addition to, a membrane proximal region that contains two leucine zipper motifs. Eleven consensus sites for N-linked glycosylation are present in the extracellular region, which may account for the difference in apparent molecular mass between the native protein and the calculated mass.

Based on the results discussed above, it would be apparent to one of ordinary skill in the art that variances in receptor sequences or in toxin binding affinities or in receptor expression may render different levels of toxin susceptibility or resistance. Furthermore, the receptor of the present invention may be used to generate transgenic organisms by methods well known in the art.

To investigate the mode of action of BT toxin, a mammalian heterologous cell culture system was chosen for several reasons. First, BT Cry1A toxins have shown no toxic effect on any mammalian cell lines studied to date. This characteristic is in contrast to most available insect cell lines, which exhibit variable degrees of sensitivity to toxin (Kwa et al., 1998). Second, the use of a mammalian cell would allow the determination of whether the receptor, independent of any associated protein in an insect cell line, would mediate toxicity.

When introduced into mammalian COS-7 cells, the cloned cDNA expressed BT-R_2 that was detected by western blot analysis using BT-R_1 antisera. The expressed receptor was displayed on the cell surface and detected with polyclonal antibodies raised against *M. sexta* BT-R_1. These results suggest that the protein expressed by the PBW BT-R_2 cDNA is similar to the natural protein found in the insect midgut.

The possibility of using COS-7 mammalian cells transfected with a receptor for BT toxins as a model system for assessing the cytotoxicity of the Cry1A toxin was determined. The surface receptor clearly was able to bind to the Cry1Ab toxin, which was detected by immunofluorescent labeling using Cry1Ab antibodies (data not shown). These results indicate that the binding site of the receptor must assume its native conformation. Significantly, intensively labeled vesicles in the methanol fixed transfected COS-7 cells were observed when the cells were incubated with BT-R_1 antiserum (data not shown). This observation indicates that vesicles, which form normally in the cell endocytosis/exocytosis pathway, contain the BT-R_2 proteins. In addition, this result shows that the receptor is not only expressed on the cell surface, like its native counter part

in the insect midgut, but also is recycled normally by the cell.

Microscopy of the transfected COS-7 cells treated with Cry1Ab toxins for various times demonstrated significant cytopathological patterns. The cytopathological changes observed under the fluorescent microscope included disruption of the plasma membrane, cell swelling, disintegration and death of the cells. The symptoms were obtained in the presence of 0.6 μg/ml Cry1Ab for 2 hr. In contrast, no cytopathological effects were revealed for cells transfected with vector alone and subsequently treated with toxin. Clearly, there is a distinct correlation between toxin binding to the surface receptor and toxicity to the cells.

The cytological appearance and ultrastructure of the midgut cells of *M. sexta* and other lepidopteran larvae, after intoxication with preparations of BT, have been reported extensively by several authors (Bravo et al., 1992). Histopathological studies on *M. sexta* midgut demonstrated pathological behavior for Cry1A on midgut epithelial cells (columnar cells) (Midhoe et al., 1999). These investigators demonstrated that the epithelial cells of the midgut swell shortly after ingestion of the BT toxin. Eventually, the

epithelial cells burst and released their cytoplasmic contents into the midgut lumen.

The present observations on the intoxicated transfected COS-7 cells are in complete agreement with these reports, which demonstrates that the toxin acts similarly in both systems. Furthermore, it should be apparent to one of ordinary skill in the art that cells expressing transfected molecules of the BT toxin receptor as well as cells expressing a natural form of the receptor may be used to asses the level of cytotoxicity and mode of action of toxins.

Lepidopteran insects generally express high molecular weight binding proteins for the Cry1A toxins that range in size from 160 to 220 kD (Martinez-Ramirez 1994; Vadlamudi et al.; 1993, Oddouet al., 1993; Nagamatsu et al., 1998a; Ihara et al., 1998). Two of these proteins, in addition to the 200 kD pink bollworm receptor, have been cloned and sequenced: the $BT\text{-}R_1$ 210 kD cadherin-related receptor from *M. sexta* (Vadlamudi et al., 1995) and the 175 kD cadherin-related from *B. mori* (Nagamatsu et al., 1998a). Interestingly, these two proteins have 60-70% identity and 80% similarity between themselves.

P. gossypiella expresses a high-affinity and a low-affinity binding protein for at least one Cry1A toxin,

Cry1Ac. The high-affinity receptor is a cadherin-related protein with a large molecular mass. One of the most important conserved regions may be the signature sequence. The signature sequence contains the sequence (READ), which is believed to be responsible for toxin binding due to the presence of two negatively charged amino acids that bind to two arginines in the toxin binding site. Supporting evidence comes from the immunoblot analysis for clones PBW-866, which contains the proposed signature sequence, and PBW-421, which does not include the signature sequence. To further define the minimum binding fragment, truncation peptides were tested for their ability to bind toxin (Figure 4). The minimum binding fragment contains the "READ" signature sequence and consists of amino acids 1269 to 1367.

The information provided herein is necessary for understanding the molecular biology of the toxin receptor in the pink bollworm and to engineer more effective toxins in terms of longer persistence in the field, higher toxicity, and preclusion of resistance development. This information will facilitate understanding of Cry toxin receptor interactions in other economically important insect crop pests.

EXAMPLE 1 SPECIFICITY OF PURIFIED TOXINS

Recombinant protoxins Cry1Aa, Cry1Ab, and Cry1Ac (*Bacillus* Genetic Stock Center, Ohio State University) were prepared from *E. coli* JM-103 and trypsinized essentially as described by Lee et al. *J. Biol. Chem.* (1992) 267: 3115. In addition, the soluble trypsinized 60 kD toxins were subjected to FPLC NaC1 salt gradient purification over an HR-5/5 Mono-Q anion exchange column (PHARMACIA™) prior to quantitation, radio-iodination, and use in bioassays. Cry3A crystal protein from *B. thuringiensis* subsp. *tenebrionis* was solubilized in 3.3 M NaBr and treated with papain, and the resulting 67 kD toxin was purified by the method of Li et al. *Nature* (1991) 353: 815. The 65 kD Cry11A toxin was isolated from *B. thuringiensis* subsp. *israelensis* via solubilization as described by Chilcott et al. *J. Gen. Micro* (1988) **134**: 1551 and further purified by anion-exchange FPLC. All toxin protein quantitations were performed using the bicinchoninic acid method (PIERCE CHEMICAL™) with Bovine Serum Albumin (BSA, Fraction V) as a standard.

Pink bollworms were obtained from the USDA PINK BOLLWORM REARING FACILITY™ (PBWRF, Phoenix, AZ). An artificial diet was obtained from SOUTHLAND PRODUCTS INC.™, Lake Village, AR. The diet was reconstituted in boiling water and cooled

to 55°C. Each Cry toxin was thoroughly mixed in the warm liquid diet and bioassay cups were filled with 20 ml of diet. After cooling and drying, 10 neonate larvae were placed in each cup and the cups were immediately capped. The method of Watson, et al., *Beltwide Cotton Conference*, Memphis, Tenn. (1995) was used to determine the toxicity of trypsin-activated toxins against first-instar larvae of *P. gossypiella*. Generally, four replicates of six cups were prepared for each dose. Cups were incubated at 30° C for 21 days, the length of time necessary for more than 95% of normal *P. gossypiella* to reach pupation. At the end of 21 days, the diet cups were examined and the numbers of larvae and numbers of pupae or adults in each cup were recorded.

The specific toxicities of purified Cry1Aa, Cry1Ab, Cry1Ac, Cry3A and Cry 2A tested using neonate *P. gossypiella* larvae are shown in Figure 3B. It was determined that all three Cry1A toxins are highly toxic, with LC_{50} values ranging from 25-45 ng/cm^3 of artificial diet. Cry3A (considered toxic to coleopteran or beetle insects) and Cry IIA (considered toxic to dipteran insects, especially mosquitoes) were not toxic to *P. gossypiella* larvae at the highest concentrations tested (2000 ng/cm^3).

EXAMPLE 2 CHARACTERIZATION OF THE BT-R_2 RECEPTOR

Early fourth-instar larvae were kept on ice for 1 hr and midguts were surgically removed from the larvae. BBMW were prepared from midgut tissues by the differential magnesium precipitation method of Wolfersberger, et al., *Comp. Biochem. Physiol.* (1987) 86A: 30, in the presence of protease inhibitors (5 mg/ml pepstatin, antipain, aprotonin, leupeptin, 1 mM PMSF, and 5 mM benzamidine). The final pellet was resuspended in buffer A (300 mM mannitol, 5 mM EGTA, and 17 mM Tris-HCl, pH 7.5) containing the protease inhibitors, flash frozen in liquid nitrogen, and stored at -85°C.

Cry toxins were radioiodinated using the chloramine T method (Hunter and Greenwood, *Nature* (1962) **194**: 495, with ^{125}I-Na (NEN DUPONT ™). Ten μg of toxin were mixed with 5 μ1 of ^{125}I-Na (0.5 mCi) in 100 μ1 of $NaHPO_4$ buffer (0.5 M, pH 7.4) with 25 μ1 of Chloramine T (4 mg/ml). The reaction mixture was agitated for 20-25 seconds at 23°C and the reaction was stopped by adding 50 μ1 of $Na_2S_2O_5$ (4.4 mg/ml). Free iodine was removed by gel filtration on an EXCELLULOSE ™ desalting column (PIERCE ™) equilibrated with PBS containing 10 mg/ml BSA.

TOXIN BINDING ASSAYS.

Both homologous and heterologous competition inhibition binding assays were performed as described by Keeton and Bulla (1997). A total of 25 μg of BBMV were incubated with 1.2 nM ^{125}I-Cry1Ac toxin in the presence of increasing concentrations (0-1000 nM) of the appropriate unlabeled homologous toxin (Cry1Ac) or heterologous toxins (Cry1Aa, Cry1Ab, Cry3A, and Cry11A). Incubations were in 100 μl of binding buffer (PBS/0.2% BSA) at 25°C for 30 min. Radiolabeled and unlabeled toxins were mixed together before adding them to the BBMV. Unbound toxins were separated from BBMV-bound toxin by centrifugation at 14,000 x g for 10 min. The pellet containing bound toxin was washed three times in ice cold binding buffer by gentle vortexing and radioactivity in the final pellet was measured using a BECKMAN GAMMA 5500 ™ counter. Binding data were analyzed by the PRISM ™ program (GRAPHPAD SOFTWARE INC.™, San Diego).

Competition inhibition binding of ^{125} I-Cry1Ac toxin to *P. gossypiella* was carried out in the presence of increasing concentrations of unlabeled Cry1Ac, Cry1Ab, Cry1Aa, Cry3A and Cry11A toxins. Homologous competition binding assays were performed with iodinated Cry1A toxins and various

concentrations of the corresponding unlabeled toxin. The binding site concentration (B_{max}), and dissociation constant (K_d) of labeled toxins were calculated from three separate experiments. The equilibrium binding parameters were estimated by analyzing the data with the PRISM™ computer program.

RADIOLIGAND BLOTTING.

The two hundred μg of BBMW proteins were solubilized, separated by 7.5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane as described by Francis and Bulla (1997). Blots were blocked with TBS (10 mM Tris-HCI and 0.9 % NaC1) containing 5% non-fat dry milk powder, 5% glycerol 0.5% Tween-20, and 0.025% sodium azide for 2 hr at 25°C. Blocking buffer was removed and membranes were incubated for 2 hr at 25°C in an equal volume of fresh blocking buffer containing 2 x 10^5 cpm/ml (1-1.25 nM) of ^{125}I-Cry1A toxins either in the presence or absence of unlabeled toxins. Finally, membranes were washed three times with fresh blocking buffer for 10 min each, rinsed once with TBS, dried, and exposed to Kodak X-ray film at -80° C.

To determine the specificity of binding to the 200 and 120 kD proteins, blots of PBW BBMV proteins was incubated

with ^{125}1-Cry1Ac toxin in the presence of increasing concentrations of unlabeled Cry1Ac toxin.

IMMUNOPRECIPITATION OF CRY1AB BINDING PROTEIN.

Immunoprecipitation was carried out according to Vadlamudi, et al. (1993). Twenty five μl of Cry 1Ab antiserum were added to 1 ml of protein A-Sepharose CL-4B equilibrated in washing buffer (1% Nonidet P-40, 6 mM EDTA, 50 mM Tris-HC1 and 250 mM NaC1) and mixed for 1 hr at 4°C. After washing the blot three times with washing buffer, 700 μg of Cry 1Ab toxin were added and the mixture were incubated for an additional 1 hr at 4°C and washed again three times with washing buffer. Pink bollworm BBMV proteins (6 mg) were solubilized in washing buffer containing 1% NP-40 and protease inhibitors (10 μg/ml pepstatin, antipain, aprotonin and leupeptin; 5 mM iodoacetamide; and 1 mM PMSF). Unsolubilized proteins were removed by centrifugation. Solubilized proteins were filtered through a 0.45 μm filter, added to 1 ml of Sepharose-protein A beads linked to Cry1Ab toxins, and the sample was stirred gently for 1 hr at 4°C. Sepharose beads were centrifuged and washed four times with washing buffer containing 0.25% NP-40 and 0.02% SDS. The toxin-binding protein complex was dissociated by heating in Laemmli (1970) sample buffer and the binding proteins were

Coomassie stained and detected by ligand blotting with ^{125}I-Cry1Ab and Western blot using Cry1Ab antiserum.

IMMUNODETECTION OF PINK BOLLWORM CRY1A RECEPTOR.

Immunoprecipitated proteins were transferred to a PVDF membrane, blocked with 5% nonfat dry milk in PBS buffer and incubated at 4°C overnight in the same blocking buffer containing 10 μg/ml of Cry1Ab. Unbound toxin was washed with PBS. Antibodies raised in rabbits against the 60 kD Cry1Ab toxin were diluted 1:1000 and hybridized to the membrane for 2 hr at 25°C and the blot then was washed with PBS. Peroxidase-conjugated goat anti-rabbit IgG was diluted 1:3000 in TBS blocking buffer and hybridized to the membrane for 2 hr. The membrane then was washed extensively with PBS. Visualization of the bound toxin was accomplished using the Enhanced Chemiluminescence (ECL) Western blotting detection method (AMERSHAM™).

SOUTHERN BLOT ANALYSIS.

Forty μg of PvuH digested genomic DNA from *P. gossypiella* or *M. sexta* were separated on a 0.8 % 1X TBE-agarose gel and blotted onto a nylon membrane (BIO-RAD™, ZETA-PROBE GT™). The analysis was carried out according to Sambrook, et al. *Molecular Cloning: A Laboratory Manual*,

2nd Ed. Cold Spring Harbor Laboratory, N.Y. (1989). The filter was hybridized with ^{32}P-labeled, random primed, C-terminal of BT-R_1 cDNA (HincH fragment, 0.5 kb). Filter hybridization was carried out at 42°C for 21 hr in 50 % formamide, 5X Denhardt's reagent, 1M NaCl, 2% SDS, 50 mM Tris-HC1 and 100 μg/ml of salmon sperm DNA. The filter was washed with 2X SSC, 0.5% SDS, then with 1X SSC, 0.5% SDS, then with 0.5X SSC, 0.5% SDS, followed by a fourth wash with 0.25X SSC, 0.5% SDS. Each wash was for 30 min at 42°C. Finally, the filter was rinsed in 2X SSC and exposed to Kodak X-ray film at -85°C.

ELECTROPHORETIC ELUTION OF PROTEINS.

Electrophoresis was performed in 1.5-mm-thick polyacrylamide slab gels using 7.5% acrylamide (pH 8.0). After SDS-PAGE, proteins were revealed as transparent bands with 4 M sodium acetate solution. The proteins were excised using a razor blade. Proteins in the gel strips were fixed in 50 % (v/v) methanol solution for 15 min and equilibrated twice in 0.125 M Tris-HC1 buffer (pH 6.8) and 2% 2-mercaptoethanol for an additional 15 min. Equilibration of the gel strips in the above buffer with 1% (w/v) SDS was performed as described above. The equilibrated gel strips

were inserted into a dialysis tube with a minimum amount of the buffer containing SDS (25 mM Tris, 190 mM glycine and 0.1% SDS). Electroelution was carried out essentially as described by Findlay (1990). A horizontal flat-bed mini-gel electrophoresis apparatus (BIO-RAD™) was used for electroelution at 50 V for 12 hr at 4°C. The buffer consisted of 25 mm Tris, 190 mM glycine and 0.1% SDS (pH 8.3). At the end of electrophoresis, the polarity of electrodes was changed for 30 sec to avoid adsorption of proteins onto the dialysis tubes. The buffer inside the dialysis tubes was collected and the tubes were washed three times with a minimum volume of buffer. SDS was dialyzed out and protein was concentrated by using a CENTRICON-30 micro-concentrator (AMICON).

TWO-DIMENSIONAL GEL ELECTROPHORESIS.

Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (1975). Isoelectric focusing was carried out in 2.0 mm (I.D.) glass tubes using 2.0% ampholines (pH 3.5-10; LKB/PHARMACIA™) for 9600 volt-hr. After equilibration for 10 min in buffer `O', tube gels were applied to the stacking gels on top of 8% acrylamide (pH 8.0) slab gels (14 x 14 cm). SDS slab gel electrophoresis

was carried out for 4 hr at 12.5 mA. After electrophoresis, one gel was stained with Coomassie blue and the others were transblotted onto PVDF paper overnight at 200 mA (Vadlamudi et al., 1993). The PVDF paper was blocked with powdered milk solution, incubated with ^{125}I-Cry1Ac or ^{125}I-Cry1Ab and exposed to X-ray film at -85°C.

IDENTIFICATION AND RECOVERY OF cDNA ENCODING BT-R_2.

Total RNA was prepared from the midgut tissue of fourth instar larvae of the PBW by the guanidinium thiocyanate method (Chomczynki et al. *Analyt. Biochem.* (1987) **162**: 156). Poly (A+) RNA was isolated with the POLYATRACT MRNA ISOLATION SYSTEM™ (PROMEGA™). First strand cDNA was synthesized using oligo-(dT) and random hexamer primers and reverse transcriptase according to standard methodologies and used as the template for amplification by polymerase chain reaction (PCR) of desired mRNAs. Degenerate oligonucleotide primers were designed based on the conserved amnio acids between *M. sexta* BT-R_1 and *B. mori* BT-R175. Such primers were used to clone partial fragments of PBW BT-R_2.

For cloning of the PBW BT-R_2, RT-PCR was employed using fully degenerate oligonucleotide primers derived from a sequence in the membrane proximal domain conserved sequence

between *M sexta* BT-R_1 and *B. mori* BT-R175. Primers BT-R-1355U and BT-R-1209U against BT-R-1486D were applied to PBW cDNA to amplify 421-bp and 866-bp fragments. The PCR products were resolved on 1.5% agarose, gel purified, cloned into a TA cloning vector (INVITROGEN™) and transformed into *E. coli* INV∝F. The presence and identity of the correct insert was confirmed with EcoR1 digestion and DNA sequencing. The PBW-886 clone was found to contain the nucleotide sequence found in clone PBW-421. In addition, primer 1209U against 1657D was used to clone a 1373-bp fragment (PBW-1373), which represents most of the membrane proximal domain and the cytoplasmic domain. Clone PBW-287 (aa 1346-1438) is a 287 bp internal fragment from 866-bp clone and was cloned using gene specific primers P5 and P6.

Based on the sequence obtained from the partial clones, sense and antisense primers were used to clone the 3' and 5' ends of the PBW BT-R_2 clone by the 5' and 3' RACE system according to the manufacturer's instructions (GIBCO BRL™). The 5' end was amplified using gene-specific antisense primers GSP1, GSP2 and GSP3 against ABRIDGED UNIVERSAL AMPLIFICATION PRIMER™ (AUAP™) provided in the kit. The 3' end was amplified using gene primer GSP4 against AUAP™. The PCR product of the predicted size was isolated and subcloned

into TA cloning vector pCR2.1 (INVITROGEN™) and transferred into *E. coli* INV∝F. For recombinant protein expression in *E. coli*, or COS7 cells, the coding sequences for the RT-PCR clones or the full length PBW-BT-R_2 clone were recloned into the pET30 or pcDNA3.1 expression vectors and transformed into BL21 (DE3) LysS (NOVAGEN™) or COS7 mammalian cells. The *E. coli* cultures were induced using a 1 mM final concentration of IPTG for 3 hr.

The full length PBW BT-R_2 (~5.5 kb; see sequence in Figure 1 SEQ ID NO:1) was ligated into the mammalian expression vector pcDNA3.1 (INVITROGEN™) and confirmed by DNA sequencing. The molecular mass of the deduced polypeptide is 194 kD with a pI of 4.1. The receptor has an open reading frame of 1729 amino acids (Figure 2) (SEQ ID NO: 2). The amino acid sequence contains a putative signal peptide of 23 amino acid residues, a transmembrane domain of 27 residues (aa 1578-1605) and a 124-residue cytoplasmic domain. In addition, the amino acid sequence contains 12 putative cadherin motifs, 11 putative N-glycosylation sites and two leucin zipper motifs at amino acid 1541-1562 and 1578-1600. The minimum toxin binding fragment is amino acids 1269 to 1367 (Figure 4).

When the protein homology is analyzed by BLASTP, as described under definitions above, the closest paralog in the GenBank nonredundant (nr) database is the *Bombyx mori* receptor at Acc. No. JE0128 with Identities = 1034/1708 (60%), Positives = 1266/1708 (73%), Gaps = 35/1708 (2%). The next closest species was *Manduca sexta* at Acc. No. AAB33758.1 with Identities = 871/1540 (56%), Positives = 1101/1540 (70%), Gaps = 22/1540 (1%). The nucleotide sequence showed no significant homologies.

The peptide homologies amongst these three species are shown in FIGURES 5A-C where perfectly conserved residues are boxed. Peptide fragments of the SBW sequence may be used to generate specific or nonspecific antibodies. Usually, it is recommended that at least 17 amino acid peptide fragments are used to generate antibodies, however, smaller peptides may also be antigenic and sufficiently complex to be unique. In particular, the carboxyl tail (aa 1677-end) of the PBW sequence is unique to this species and can be used to generate PBW unique antibodies. Exemplary peptides that may be useful as antigens (numbered with respect to FIGURE 5, SEQ ID NO: 2) are shown as follows:

PBW Unique Peptides	Common Peptides
aa 534-544	aa 291-304
aa 697-705	aa 622-632
aa 886-895	aa 791-803
aa 1055-1066	aa 1621-1642
aa 1321-1331	
aa 1451-1461	
aa 1516-1525	
aa 1572-1582	
aa 1677-1729	

IMMUNODETECTION OF THE EXPRESSED BT-R_2 PROTEINS.

Cell lysates from the induced BL21 (DE3) LysS bacterial cultures were electrophoresed and transferred to PVDF membranes. Filters were blocked at 4°C in 50 ml of blocking buffer containing 10 ug/ml of Cry1Ab toxin. Unbound toxin was removed by PBS. Rabbit primary antibodies for the THW was removed by PBS. Rabbit primary antibodies for the THW BT-R, extracellular domain or for the FPLC-purified Cry1Ab were diluted 1:1000 in 50 ml TBS blocking buffer. The filters were incubated for 2 hr with the antiserum and washed three times with the blocking buffer. Peroxidase-conjugated goat anti-rabbit IgG was diluted to 1:2000 and incubated with

filters for 2 hr at 27°C and was developed with the enhanced chemoluminescence (ECL) detection system (AMERSHAM™).

MAMMALIAN EXPRESSION OF BT-R_2.

The PBW BT-R_2 cDNA cloned into pcDNA3.1, a mammalian expression vector (INVITROGEN™), was expressed in mammalian cells (COS-7 SV40 transformed African green monkey cells; ATCC CRL-1651) according to methods described by Keeton and Bulla, *Appl. Environ. Microbiol.* (1997) **63**: 3419. COS-7 cells (4 x 10^4/well) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) on 12 mm cover slips placed in a 24-well plate.

COS-7 cells were transfected with the construct using the LIPOFECTAMIN PLUS REAGENT™ (GIBCO BRL™). The cells were incubated for two days at 37°C in DMEM medium containing 10% FBS in a humidified atmosphere of 10%CO_2. BT-R_2 was monitored by SDS-PAGE and immunoblotting with anti-BT-R_1 or antiCry1Ab antiserum. Surface expression was detected by immunofluorescence microscopy with the anti-BT-R_1 antibodies. The effects of BT toxin on the transfected cells were demonstrated by incubating the cells in the presence or absence of Cry1Ab toxin for 2 or 4 hr and monitoring the

morphological changes by immunofluorescence microscopy using either anti-BT-R_1 or anti-Cry1Ab antibodies. Cell death is clearly demonstrated (not shown).

IMMUNOFLOURESCENCE MICROSCOPY.

COS-7 cells were grown on 12-mm glass coverslips in a 24-well plate. The cells were fixed and permeabilized either in cold methanol (-20°C) or 4% paraformaldhyde for 15 minutes at 27°C. Coverslips were rinsed three times with PBS and then blocked for 15 minutes with 1% BSA in PBS. Cells were incubated with primary antibody for 30 minutes at 27°C followed by rinsing and blocking as just described. The same incubation and washing procedures were applied to secondary antibody. Antibodies were detected with TRITC goat anti-rabbit IgG. Coverslips were mounted in FLUROMOUNT G™ and viewed with an OLYMPUS™ microscope equipped with epi-fluorescence illumination and a 40X Apochromat lens. Photography was done with an OLYMPUS SPOT™ camera.

WESTERN BLOT ANALYSIS.

Transfected COS-7 cells were washed with cold PBS, lysed in lysis buffer (50 mM Tris/HCL, 1 mM EDTA, 10 μM leupeptin) and resuspended on ice for 10 minutes. Then, 4X sample buffer was added to the cells and heated at 95°C for 5 minutes. Lysates were subjected to electrophoresis through 7.5% SDS-PAGE, and proteins were electrophoretically transferred to a PVDF filter, blocked and incubated with either anti-BT-R_1, or anti-Cry1Ab antibodies.

RESULTS: IDENTIFICATION OF ^{125}I-CRY1A BINDING PROTEINS.

BBMV proteins of *P. gossypiella* ranged in molecular size from greater than 205 kD to less than 25 kD (data not shown) as determined by SDS-PAGE. ^{125}I-labeled Cry1Aa, Cry1Ab and Cry1Ac were used in ligand blots to identify which *P. gossypiella* BBMV proteins bind the respective toxins. Proteins that had been separated by SDS-PAGE were transferred to PVDF membranes and incubated with each radiolabeled-toxin separately. ^{125}I-Cry1Aa, ^{125}I-Cry1Ab and ^{125}I-Cry1Ac bound to a protein of about 200 kD (data not shown). ^{125}I-Cry1Ac bound also to a protein band at about 120 kD. Neither Cry1Aa nor Cry1Ab bound to the 120 kD protein. The binding patterns for

all three toxins were the same under both reducing and nonreducing conditions (data not shown).

RESULTS: COMPETITION INHIBITION BINDING ASSAYS.

^{125}I-labeled Cry1Aa, Cry1 Ab and Cry1Ac were used in binding assays with *P. gossypiella* BBMV. Competition binding of ^{125}1-Cry1Ac toxin to *P. gossypiella* was carried out in the presence of increasing concentrations of unlabeled Cry1Aa, Cry1Ab, Cry1Ac, Cry3A and Cry11A toxins. Fifty-percent inhibition of Cry1Ac binding was observed at 10 nM of unlabeled Cry1Ac, 100 nM unlabeled Cry1Aa and 100 nM of unlabeled Cry1Ab. At a concentration of 1000 nm, unlabeled Cry1Ac, Cry1Ab and Cry1Aa reduced binding of iodinated Cry1Ac by 95, 82 and 80%, respectively (data not shown). Neither Cry3A nor Cry11A toxin competed for the Cry1Ac toxin binding site.

Homologous competition binding assays were performed with iodinated Cry1A toxins and various concentrations of the corresponding unlabeled toxin Cry1Aa, Cry1Ab and Cry1Ac showed high binding affinity to BBW proteins (data not shown). Fifty-percent inhibition of binding of Cry1A toxins was observed at concentrations of approximately 10 nM of the corresponding unlabeled toxin. These data indicate that each

of the three toxins binds specifically with high affinity. The binding site concentration, B_{max}, and the dissociation constant, K_d, of each toxin was calculated from the three separate homologous competition inhibition experiments by analyzing the data with the GRAPHAD computer program (Table 1). The K_d values all were similar and in the low nM range whereas the B_{max} for Cry1Ac was higher than Cry1Aa or Cry1Ab. The Hill coefficients for Cry1Aa, Cry1Ab and Cry1Ac were 0.65, 0.65, and 0.77, respectively, indicating a negative binding cooperativity for the toxins against the BBMV proteins. A single binding site model was indicated based on the nonlinear regression analysis for both Cry1Aa and Cry1Ab. Significantly, Cry1Ac, the data was best accommodated by a two binding site model with high- and low-affinity binding sites.

Results: Specificity of ^{125}I-Cry1Ac Toxin Binding in Ligand Blots.

In view of the putative "two-binding site" model predicted for the Cry1Ac toxin, radioligand blots of *P. gossypiella* BBMV proteins were carried out with ^{125}I-Cry1Ac toxin in the presence of increasing concentrations of unlabeled Cry1Ac toxin. Autoradiography of these blots revealed significant reduction in the intensity of the 200

kD band (data not shown). Indeed, it was undetectable at a Cry1Ac toxin concentration of 10 nM. In the case of the 120 kD band, however, there was virtually no reduction in the band intensity (data not shown) even at a Cry1Ac concentration of 1000 nM. In saturation binding assays, incubation of a fixed amount of each of the three ^{125}I-labeled Cry1A toxins with increasing concentrations of BBMV showed that binding reached a saturation level in each case but that the level of Cry1Ac binding was substantially higher than those of Cry1Aa and Cry1Ab. Maximum saturable binding at 400 μg/ml of BBMV was approximately 0.35, 0.05 and 1.5 ng for Cry1Aa, Cry1Ab and Cry1Ac, respectively, which represents an approximately 30-fold difference in Cry1Ac binding compared to Cry1Ab, and, it is 4 fold higher for Cry1Ac compared to Cry1Aa (data not shown).

Results: Immunoprecipitation of the Cry1Ab Binding Protein.

Immunoprecipitation experiments were performed using Cry1Ab, which has the highest binding affinity of the three toxins, to further examine the specificity of binding of the toxin to the 200 kD protein. BBMV proteins were solubilized in 1% NONIDET P-40™ and immunoprecipitated with anti-toxin-protein A-Sepharose beads. The mixture of bound material was

solubilized in SDS sample buffer containing 2-mercaptoethanol. Electrophoresis and staining of the gel with Coomassie blue revealed a protein of about 200 kDa, demonstrating selective precipitation of the 200 kD toxin-binding protein. Radioligand blotting with ^{125}I-Cry1Ab showed a band of about 200 kDa (data not shown), indicating precipitation of the same binding protein as that identified in previous ligand blot experiments. Additionally, a Western blot (data not shown) of the immunoprecipitated protein using Cry1Ab and anti-Cry1Ab polyclonal antiserum confirmed the results of the radio-ligand blot (data not shown). The low-molecular weight bands at 60 and 52 kDa correspond to the Cry1Ab toxin and the heavy chain of IgG, respectively.

RESULTS: PURIFICATION OF THE BINDING PROTEINS.

To determine whether the 200 kD band contains more than one protein, the band was excised from a 7.5% SDS polyacrylamide gel, electroeluted, dialyzed and concentrated. The concentrated protein was analyzed by two-dimensional gel electrophoresis over a pH range of 3.5-10. The protein migrated as one spot with an estimated p1 of 4.5 ± 0.2 and apparent molecular mass of 200 kDa. The purified 200 kD protein stained with Schiff's reagent (data not shown)

indicating that the binding protein is glycosylated. The 200 kD IEF spot bound ^{125}I -Cry1Ab (data not shown) corroborates the results from other immunoprecipitation studies.

RESULTS: SOUTHERN BLOT ANALYSIS.

To detect the presence of the Cry1A receptor in *P. gossypiella*, genomic DNA from both insects were hybridized against the cloned THW BT-R_1 cDNA and its 507-bp minimum binding fragment. The two probes bound intensively to the PvuH fragment of *M. sexta* genomic DNA (data not shown). There was weak hybridization to the *P. gossypiella* DNA, however, using the minimum binding probe and none with the full-length BT-R_1 probe (data not shown). These results suggest that the minimum binding fragment from *M. sexta* shares a significant level of nucleotide similarity to the Cry1A binding receptor in *P. gossypiella*, more so than to the full-length BT-R_1 receptor.

RESULTS: IMMUNODETECTION OF NATIVE AND CLONED PBW BT-R_2 USING BT-R_1 ANTIBODIES.

To confirm the relatedness of the cloned PBW fragment to the THW BT-R_1 and its ability to bind toxin, it was subcloned into a pET30 expression vector. The native PBW

BBMV proteins and the expressed proteins from clones PBW-287, -421 and -866 were resolved by SDS-PAGE, transferred to a PVDF membrane and incubated with either anti-BT-R_1 serum or Cry1Ab toxin followed by antiserum to the toxin. The results reveal that BBMV contain a 200 kD protein that interacts with THW BT-R_1 antiserum (data not shown). In addition, clones PBW-287, -421 and -866 which express proteins of about 15, 21 and 32 kD, respectively, also cross-reacted with BT-R_1 antiserum. The 32 kD clone, however, was the only protein to bind toxin, whereas no detectable binding was observed with the 21 kD protein (data not shown). These results confirm the sequence relatedness of PBW BT-R_2 to THW BT-R_1 and demonstrate that the 32 kD protein contains the toxin-binding site of the receptor.

RESULTS: SPECIFICITY OF TOXIN BINDING TO THE CLONED RECEPTOR.

The specificity and affinity of toxin binding to the receptor fragment (PBW-866) was determined using competition ligand blot analysis. The expressed 32 kD protein was transferred to PVDF membranes and incubated with ^{125}I-Cry1Ab in the absence or presence of increasing concentrations of unlabeled Cry1Ab toxin. Autoradiography revealed significant reduction in the intensity of the 32 kD band to an

undetectable level in the presence of 500 nM unlabeled Cry1Ab toxin (data not shown). Bound ^{125}I toxin was quantitated with a gamma counter and the BIO-RAD IMAGER™ analysis system was used to calculate the binding affinity of toxin to the expressed fragment. The binding affinity (~17nM) of the toxin was similar to the calculated value (Table 1) for BBMV. These results demonstrate that Cry1Ab binds specifically with high affinity to PBW BT-R_2 866. Other truncation fragments were also tested, and it was determined that the minimum binding fragment consists of amino acids 1269 to 1367.

RESULTS: EXPRESSION OF PBW BT-R_2 IN COS-7 CELLS.

PBW BT-R_2 cDNA was subcloned into the mammalian expression vector pcDNA3.1 (INVITROGEN™) and transfected into COS-7 cells. Protein encoded by the PBW BT-R_2 cDNA was expressed as a membrane protein capable of binding Cry1Ab toxin. Membranes isolated from transiently transfected COS-7 cells were solubilized, electrophoresed, and immunoblotted either with Cry1Ab toxin and its antiserum or with BT-R_1 antiserum directly. The expressed 220 kD receptor bound Cry1Ab toxin and cross-reacted with BT-R_1 antiserum. No interaction to vector transfected cells was observed.

Expression of BT-R_2 receptor on the cell surface was shown by fixing the cells in methanol or paraformaldehyde and incubating first with anti-BT-R_1 serum, and then with TRITC IgG secondary antibodies. Transfected cells portrayed bright surfaces due to the binding of BT-R_1 antibodies to the cell surface clearly showing that the PBW BT-R_2 receptor is expressed on the cell surface.

The surface-expressed PBW receptor binds toxin and kills the cells. Transfected cells were incubated with Cry1Ab toxin for 2 or 4 hr, washed, fixed and incubated first with anti-Cry1Ab antiserum, and then with TRITC IgG secondary antibodies. As shown by immunofluorescence microscopy, BT-R_2 expressing COS-7 cells bound the toxin, whereas cells transfected with vector alone did not show any surface binding of toxin. Incubation of cells expressing PBW BT-R_2 with toxin for 2 or 4 hr showed significant morphological changes which include loss of cell integrity, loss of cell cytoplasm and complete disintegration of the plasma membrane and cell death.

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While this invention has been described with reference to illustrative embodiments, this description is not intended to be construed in a limiting sense. Various modifications and combinations of illustrative embodiments, as well as other embodiments of the invention, will be apparent to persons skilled in the art upon reference to the description.

It is therefore intended that the appended claims encompass such modifications and enhancements.